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Opsonization of *Cryptococcus neoformans* by Human Immunoglobulin G: Masking of Immunoglobulin G by Cryptococcal Polysaccharide

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Previous studies have shown that attachment of non-encapsulated cryptococci to macrophages is highly dependent on opsonizing immunoglobulin G (IgG) and that cryptococcal polysaccharide inhibits the attachment phase of phagocytosis. We investigated various mechanisms by which cryptococcal polysaccharide might interfere with the opsonizing action of IgG. Cryptococcal polysaccharide did not appreciably prevent binding of opsonizing IgG to the yeast. Furthermore, cryptococcal polysaccharide acted as a noncompetitive inhibitor with respect to the opsonizing action of IgG. These experiments suggested that cell wall-bound IgG is masked in some manner such that it is unable to participate in Fc-mediated phagocytosis. This appeared to be the case, since cryptococcal polysaccharide inhibited agglutination of IgG-opsonized yeast cells by antiserum to IgG. There was good dose-response correlation between the amount of polysaccharide needed to inhibit phagocytosis of non-encapsulated *Cryptococcus neoformans* and the amount of polysaccharide needed to prevent agglutination of IgG-opsonized cryptococci by antiserum to IgG. The ability of cryptococcal polysaccharide to prevent agglutination of IgG-opsonized cryptococci by antiserum to IgG was lost if dextran, a substance known to enhance agglutination of several particles, was incorporated into the medium.

Cryptococcal polysaccharide prevents attachment of *Cryptococcus neoformans* to macrophages (6). As a consequence of this inhibition, phagocytosis is blocked. In the accompanying paper (7), we report that IgG is the opsonin with primary responsibility for attachment of the yeast to macrophages. Thus, interference with the opsonizing action of IgG is one mechanism by which cryptococcal polysaccharide might inhibit phagocytosis of the yeast. Cryptococcal polysaccharide might prevent binding of immunoglobulin G (IgG) to the yeast by competing with IgG for binding sites on the yeast or by presenting a barrier that prevents binding of IgG to the yeast. Alternatively, cryptococcal polysaccharide might have no effect on binding of IgG to the yeast, but the capsule could mask the yeast-bound IgG such that opsonizing IgG cannot effectively participate in Fc-mediated phagocytosis. The present study was undertaken (i) to determine whether cryptococcal polysaccharide prevents binding of IgG to the yeast by either a competitive or a noncompetitive mechanism and (ii) to determine whether cryptococcal polysaccharide produces a barrier around the yeast that masks the presence of opsonizing IgG.

MATERIALS AND METHODS

Yeast isolates and soluble polysaccharide. *C. neoformans* strain 613 is an encapsulated isolate. *C. neoformans* strain 602 is a non-encapsulated strain that has surface receptors for the soluble polysaccharide; therefore, addition of purified polysaccharide allows experimental variation in the extent of encapsulation on the yeast (4). The characteristics of these strains have been described elsewhere in detail (4, 5). Organisms used in phagocytosis assays were Formalin killed (6) and used as a suspension in Hanks balanced salt solution (HBSS; GIBCO, Grand Island, N.Y.) containing antibiotics (100 U of penicillin and 100 μg of streptomycin per ml; GIBCO) and buffered with sodium bicarbonate to pH 7.2.

The procedure for purification of cryptococcal polysaccharide has been described previously (5). Polysaccharide was prepared for use as a saline solution.

Phagocytosis assays. Unstimulated peritoneal macrophages were obtained from 6- to 8-week-old Swiss mice (Microbiological Associates, Walkersville, Md.). The procedure for collection and culture of macrophages has been described previously (6). Monolayers were prepared in four-chamber tissue culture chamber/slides (model 4804; Lab Tek Products, Naperville, Ill.) and incubated for 24 to 48 h at 37°C in 2.6% CO_2 before use. Each monolayer contained approximately 2.5×10^5 macrophages.

For phagocytosis assays, the culture medium was decanted, and each monolayer was washed two times with warm (37°C) HBSS. A test yeast suspension was warmed for 2 min at 37°C in a water bath, and 1 ml was immediately added to each chamber. The yeast suspensions consisted of: (i) 10^6 yeast cells; (ii) a source of opsonin; (iii) when required by an experimental protocol, 0.25 ml of cryptococcal polysaccharide in saline; and (iv) enough HBSS to give a final volume of 1 ml. Phagocytosis of yeast cells was determined after incubation of monolayers with yeast cells for 1 h at 37°C. Slides were washed, fixed, stained, and examined as described previously (6). The percentage of macrophages with ingested yeasts (percent phagocytosis) or the mean number of ingested yeasts per macrophage (phagocytic index) was determined. Results presented are mean values from at least four replications.

Serum and serum components. Pooled normal human serum (GIBCO; lot no R0555100) was used as an opsonin source. IgG was isolated from normal human serum by diethylaminoethyl (DEAE)-cellulose chromatography (16). Goat antiserum monospecific for the heavy (gamma) chains of human IgG was obtained from Meloy Laboratories, Inc., Springfield, Va. Human IgG was labeled with ^{125}I by the lactoperoxidase method (7, 8). Binding of ^{125}I-labeled IgG to *C. neoformans* was determined as previously described (7).

Agglutination of opsonized cryptococci by antiserum to IgG. Cells of strain 602 (2.5×10^6) in 0.1 ml of saline or saline containing dextran T-500 (Pharmacia Fine Chemicals, Uppsala, Sweden) were incubated in tubes (10 by 75 mm) for 20 min at room temperature with 12.5 μg of human IgG in 0.1 ml of saline or saline containing dextran and 0.1 ml of saline or saline containing cryptococcal polysaccharide. Goat antiserum monospecific for the heavy (gamma) chain of human IgG (0.1 ml of a 1:4 dilution in saline) was added and incubated for an additional 20 min at room temperature. The tubes were centrifuged, and agglutination was determined by macroscopic examination of sedimentation patterns and by microscopic examination for yeast agglutination.

RESULTS

Binding of IgG to *C. neoformans*. An initial experiment was done to determine whether similar amounts of opsonizing IgG could bind to encapsulated and non-encapsulated cryptococci. Cells (10^7) of strain 613 or strain 602 were incubated with various amounts of ^{125}I-labeled IgG for 30 min at room temperature. The cells were washed eight times with 0.05 M phosphate buffer (pH 7.5), and the amount of adherent IgG was determined. The results (Fig. 1) showed that cells of strain 602 bound approximately twice as much IgG as did cells of strain 613.

Reduced binding of IgG to encapsulated cryptococci could be due to: (i) competition by cryptococcal polysaccharide for IgG receptors on the yeast surface, (ii) inability of IgG to fully penetrate the capsule, or (iii) differences between

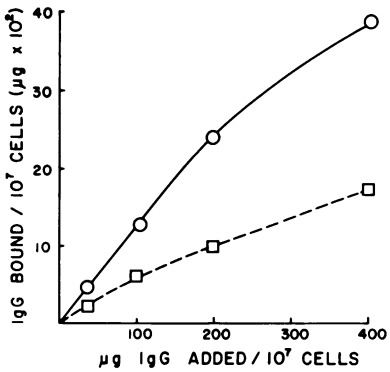

FIG. 1. *Binding of ^{125}I-labeled human IgG to cells of strain 602 (○) or strain 613 (□).*

cryptococcal strains 602 and 613 in the density or specificity of antigenic determinants on the yeast. As a consequence, the effect of purified cryptococcal polysaccharide on binding of ^{125}I-labeled IgG to the non-encapsulated strain 602 was determined. This experimental model eliminates some of the problems inherent in comparisons of encapsulated and non-encapsulated isolates. There are no strain differences, and a thick polysaccharide capsule is avoided since small amounts of cryptococcal polysaccharide are added to strain 602. Various amounts of ^{125}I-labeled IgG were added to cells of strain 602 or to cells of strain 602 that had been preincubated with 1.0 or 10 μg of cryptococcal polysaccharide per 10^6 yeast cells. Previous studies showed that these concentrations of polysaccharide bound to the yeast and were sufficient to inhibit phagocytosis of strain 602 (6). Yeast cells were incubated for 30 min at room temperature with ^{125}I-labeled IgG in the presence or absence of cryptococcal polysaccharide, and the amount of adherent IgG was determined. The results (Fig. 2) showed that within the concentrations of polysaccharide studied, cryptococcal polysaccharide did not inhibit binding of ^{125}I-labeled IgG to *C. neoformans* strain 602.

Masking of IgG. The previous experiment suggested that cryptococcal polysaccharide did not compete with IgG for binding sites on the yeast. This result was confirmed by a bioassay of the effects on phagocytosis of strain 602 of a constant amount of cryptococcal polysaccharide and a varying IgG concentration. We hypothesized that if cryptococcal polysaccharide competed with IgG for binding sites on the yeast, the phagocytosis-inhibiting properties of cryptococcal polysaccharide should decrease at increasing concentrations of opsonizing IgG. To test this hypothesis, yeast cells were mixed with: (i) human IgG at concentrations ranging from 8

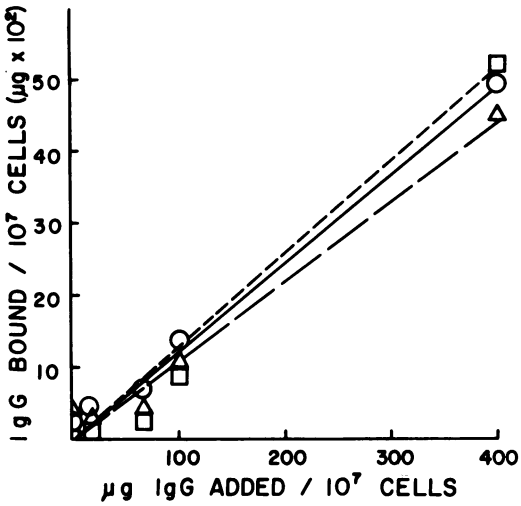

FIG. 2. *Effect of saline (○) or 1.0 μg (□) or 10 μg (△) of cryptococcal polysaccharide per 10^6 cells on the binding of ^{125}I-labeled IgG to strain 602.*

to 2,000 μg of IgG per 10^6 cells or whole human serum diluted to contain IgG at the specified concentrations and (ii) no polysaccharide or 0.5 or 2.0 μg of cryptococcal polysaccharide per 10^6 yeast cells. The yeast cells were then added to monolayers and incubated for 1 h at 37°C. The results of experiments using whole serum (Fig. 3) or purified IgG (Fig. 4) as opsonins showed that considerable inhibition of phagocytosis by cryptococcal polysaccharide occurred regardless of the concentration of opsonizing IgG. A diagnostic double reciprocal plot of 1/(phagocytic index) versus 1/(opsonin concentration) was also used to evaluate the phagocytosis data, since the curves shown in Fig. 3 and 4 could be presented as linear graphs (insets, Fig. 3 and 4). Furthermore, such curves allow an extrapolation of the lines to high levels of opsonin concentrations that cannot be achieved experimentally. We expected that as 1/(opsonin concentration) approached zero, i.e., as the opsonin concentration became very great, 1/(phagocytic index) should be identical for both the inhibited and the noninhibited curves if cryptococcal polysaccharide acts as a competitive inhibitor for binding of opsonizing IgG to the yeast. The experimental results (insets, Fig. 3 and 4) showed that extrapolation of the inhibition curves to the *y* axis provided values for 1/(phagocytic index) that were markedly different from the uninhibited curves. Thus, inhibition of phagocytosis by cryptococcal polysaccharide could not be overcome by increasing the opsonin concentration, suggesting that the polysaccharide inhibited phagocytosis in the manner of a typical noncompetitive inhibitor.

The previous experiments suggested that opsonizing IgG is masked or inhibited in some way such that the immunoglobulin is unable to participate in Fc-mediated phagocytosis. The effect of cryptococcal polysaccharide on agglutination of IgG-opsonized 602 [602(IgG)] by monospecific antiserum to IgG heavy chains was used to determine whether cryptococcal polysaccharide masks opsonizing IgG. A total of 2.5×10^6 cells of strain 602 were incubated with 12.5 μg of IgG, and various amounts of cryptococcal polysaccharide were added. This concentration of IgG

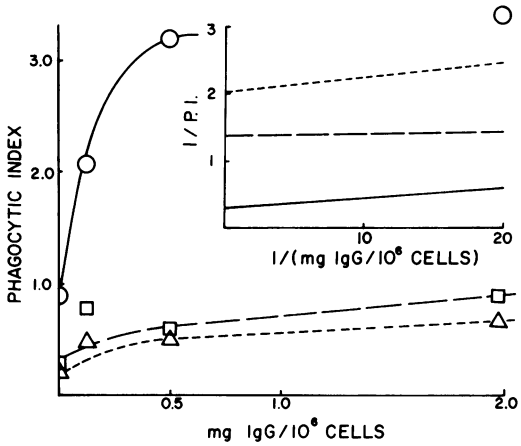

FIG. 3. *Effect of various concentrations of normal human serum upon the phagocytosis-inhibiting properties of a constant amount of polysaccharide. Phagocytosis of C. neoformans 602 in the absence of polysaccharide (○——○) and in the presence of cryptococcal polysaccharide at final concentrations of 0.5 μg/ml (□— —□) or 2.0 μg/ml (△- - -△) is shown.*

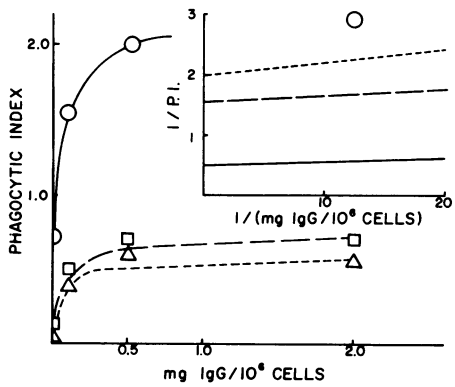

FIG. 4. *Effect of various concentrations of normal human IgG upon the phagocytosis-inhibiting properties of a constant amount of polysaccharide. Phagocytosis of C. neoformans 602 in the absence of polysaccharide (○——○) and in the presence of cryptococcal polysaccharide at a final concentration of 0.5 μg/ml (□— —□) or 2.0 μg/ml (△- - -△) is shown.*

was chosen because previous experiments (7) had shown that greater amounts of IgG would directly agglutinate 602. Agglutination of polysaccharide-treated cells by anti-IgG was determined as described in Materials and Methods and was compared with phagocytosis of polysaccharide-treated 602(IgG). Yeast cells used in phagocytosis experiments were opsonized with 5 mg of IgG per 10^7 yeast cells. The results (Fig. 5) showed that polysaccharide concentrations of 80 and 8.0 $\mu g/10^6$ cells inhibited phagocytosis of 602(IgG) and inhibited agglutination of 602(IgG) by antiserum to IgG. At lesser concentrations of cryptococcal polysaccharide (0.8 and 0.08 μg), there was good correlation between the increase in percent phagocytosis and increased agglutination of 602(IgG) by antiserum to IgG.

Dextran has been shown to enhance the ability of IgG to agglutinate particulate antigens (9, 10); therefore, an experiment was done to determine the effect of dextran on inhibition of agglutination by cryptococcal polysaccharide. 602(IgG) were incubated with cryptococcal polysaccharide (8.0 $\mu g/10^6$ cells) in the presence of various concentrations of dextran. Antiserum to human IgG was added, and agglutination was determined as described in Materials and Methods. The results (Table 1) showed that dextran had a definite effect on inhibition of agglutination by cryptococcal polysaccharide. Cryptococcal polysaccharide inhibited agglutination of 602(IgG) in the absence of dextran and at 0.08% dextran, but cryptococcal polysaccharide was unable to inhibit agglutination at higher dextran concentrations.

DISCUSSION

This study was undertaken to consider various mechanisms by which cryptococcal polysaccharide might inhibit phagocytosis of *C. neoformans*. Since cryptococcal polysaccharide inhibits the attachment stage of phagocytosis (6), and IgG is a primary factor in attachment of non-encapsulated cryptococci to macrophages (7), the interaction between cryptococcal polysaccharide and opsonizing IgG became a focal point in our investigation. Cryptococcal polysaccharide might prevent binding of opsonizing IgG to the yeast surface or it might prevent the interaction of yeast-bound IgG with Fc receptors on the macrophage surface. Similar hypothetical mechanisms have been proposed to explain the antiphagocytic action of the *Staphylococcus aureus* capsule (15).

Inhibition by polysaccharide of binding of opsonizing IgG to the yeast cannot account for the phagocytosis-inhibiting properties of cryptococcal polysaccharide. Encapsulated cryptococci

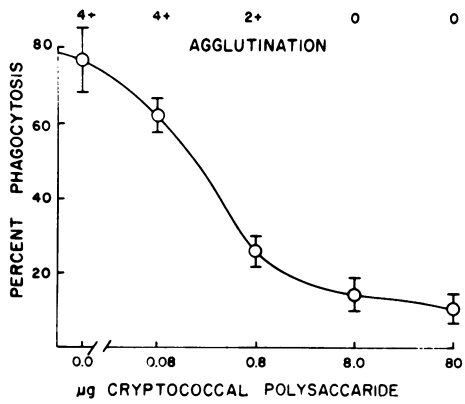

FIG. 5. *Comparison of the polysaccharide concentrations needed to inhibit phagocytosis of 602(IgG) and inhibit agglutination of 602(IgG) by antiserum to IgG heavy chains. Mean ± standard deviation.*

bound approximately half the amount of opsonizing IgG as did non-encapsulated cryptococci. Previous studies in our laboratory have shown that IgG is opsonic over wide ranges of IgG concentrations (7); therefore, a 50% reduction in the binding of IgG cannot account for the profound inhibition of phagocytosis observed with encapsulated cryptococci. Similar results were obtained when we examined the effect of cryptococcal polysaccharide on binding of IgG to non-encapsulated cryptococci. Cryptococcal polysaccharide had no effect on the binding of IgG to non-encapsulated cryptococci at concentrations of polysaccharide known to inhibit phagocytosis of the yeast (4). These results are in agreement with an earlier report by Stinson and van Oss (13) that the presence of a capsule on *S. aureus* or species of *Streptococcus* does not prevent binding and may actually potentiate binding of IgG to these bacteria.

A bioassay of the effects of various IgG concentrations on the phagocytosis-inhibiting properties of cryptococcal polysaccharide showed that inhibition of phagocytosis occurred regardless of the opsonin concentration. A double reciprocal plot of 1/(phagocytic index) versus 1/(opsonin concentration) was used to further analyze the effect of opsonin concentration on inhibition of phagocytosis. Similar double reciprocal plots have been used with varying degrees of success by other investigators (3, 4, 11, 12, 14) to evaluate several aspects of the phagocytic process. The results of such an analysis showed that inhibition of phagocytosis was not appreciably abrogated by high opsonin concentrations. Thus, cryptococcal polysaccharide does not appear to act as a competitive inhibitor of binding of IgG to the yeast cell, since competitive inhi-

TABLE 1. *Effect of dextran on inhibition by cryptococcal polysaccharide of agglutination of 602(IgG) by antiserum to IgG*[a]

Anti-IgG	Agglutination							
	10%[b]	5%[b]	1.25%[b]	0.31%[b]	0.08%[b]	0[b]	Uninhibited control[c]	Unopsonized 602
Present	4+	4+	4+	4+	0	0	4+	0
Absent	2+	0	0	0	0	0	0	0

[a] A total of 2.5×10^6 cryptococci were incubated with 12.5 μg of human IgG in the presence or absence of 20 μg of cryptococcal polysaccharide. Anti-human IgG or saline was added, and agglutination was determined macroscopically on a scale of 0 to 4+.

[b] Dextran concentration.

[c] Agglutination of 602(IgG) by anti-human IgG was determined in the absence of cryptococcal polysaccharide.

bition should have produced an inhibition that decreased substantially at high concentrations of IgG.

Since IgG binds to both encapsulated and non-encapsulated cryptococci, it must bind to encapsulated cells in a manner that makes it unable to participate in Fc-mediated phagocytosis. One likely mechanism is that cryptococcal polysaccharide acts as a barrier between cell wall-associated IgG and the macrophage Fc receptors, a mechanism that was first proposed by Stinson and van Oss (13) and more recently suggested by Wilkinson et al. (15). The data in Fig. 5 show that this is in fact the case. Agglutination of 602(IgG) by antiserum to IgG heavy chains was used as an assay for the availability of IgG at the surface of *C. neoformans*. Presumably, if 602(IgG) is agglutinated by anti-IgG, the Fc fragments would also be available for interaction with macrophage Fc receptors. Conversely, if 602(IgG) cannot be agglutinated by antiserum to IgG heavy chains, it is not likely that the Fc fragments would be available for participation in phagocytosis. Pollack et al. (9) have estimated that 7S agglutinating antibody bridges a distance of 24 to 25 nm. Thus, a polysaccharide-induced barrier of greater than 12 nm plus the length of an IgG molecule should inhibit agglutination of 602(IgG) by antiserum to IgG. The data in Fig. 5 showed that cryptococcal polysaccharide does, indeed, inhibit agglutination of 602(IgG) by antiserum to IgG. Furthermore, there was good correlation between the amount of polysaccharide needed to present a barrier that prevents agglutination and the amount of polysaccharide needed to inhibit phagocytosis of the yeast.

The effect of dextran on the ability of polysaccharide to prevent agglutination of 602(IgG) by antiserum to IgG suggests at least one mechanism by which cryptococcal polysaccharide might prevent agglutination. Pollack et al. (9) have argued that potential energy barriers are crucial factors in agglutination, and electrostatic barriers exist for erythrocytes above which agglutination by antibody cannot occur. Pollack et al. (9) demonstrated that incorporation of polymers such as dextran into the medium reduced the electrophoretic mobility of erythrocytes and concomitantly facilitated hemagglutination by antiserum. The uronic acid residue of cryptococcal polysaccharide imparts a negative charge to the molecule; therefore, it is quite possible that the polysaccharide could increase the electrostatic potential surrounding 602(IgG) such that the cells cannot be agglutinated by antiserum to IgG. If, indeed, the action of dextran is a reduction in zeta potential (9, 10), the data would suggest that cryptococcal polysaccharide presents a potential energy barrier around the yeast cell that contributes to masking of IgG. Unfortunately, the action of dextran on agglutination reactions is not completely understood, and a putative role for surface charge in inhibition of agglutination by cryptococcal polysaccharide must be considered very tentative. Brooks and Seaman (1) have suggested that the action of dextran is due to simultaneous adsorption of polymer molecules to two cells, forming links between them. Other explanations are also possible.

Data presented in this paper suggest that cryptococcal polysaccharide produces a barrier that masks opsonizing IgG on the yeast cell. Curtis (2) has stated that electrostatic forces and steric effects are important factors in determining whether or not adhesion between cells will occur. It is attractive to speculate that the physical barrier presented by the polysaccharide or a polysaccharide-induced electrostatic potential near the surface of the yeast prevents specific (IgG-mediated) and nonspecific recognition of the yeast.

ACKNOWLEDGMENTS

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